THE GROWTH AND SPORULATION OF THE BENIGN AND MALIGNANT TERTIAN MALARIAL PARASITES IN THE CULTURE TUBE AND IN THE HUMAN HOST

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PREFATORY NOTE BY SIR RONALD ROSS

Researches on the cultivation of the parasites of malaria in Liverpool were commenced some time ago at my suggestion by Dr. Sinton, and then, with better success, by Drs. J. G. Thomson and McLellan, and by Dr. D. Thomson. We are greatly obliged to Sir Edwin Durning-Lawrence, Bart., for giving us the services of Dr. J. G. Thomson for this important enquiry.

21*st May*, 1913.

Ronald Ross.

INTRODUCTION

The successful cultivation of malarial parasites was first announced by Bass and Johns (1912). Since then several workers, Thomson and McLellan (1912), J. G. Thomson and D. Thomson (1913) and Ziemann (1913) have successfully repeated these cultivation experiments. This achievement has led the way to new discoveries regarding the malarial parasite, and suggests that it may be possible to cultivate *in vitro* any protozoal parasite however specialised it may be.

So far, only the asexual generation of the malarial parasite has been grown *in vitro*. The next step is to cultivate the sexual generation as it occurs in the human host and in the mosquito. We have attempted both, but so far without definite success. We have no doubt, however, that this will also be accomplished sooner or later. In 1911, J. G. Thomson and Sinton successfully cultivated the human trypanosome, and the development they obtained was apparently that which takes place in the stomach of the tsetse fly. Joukoff (1913) states that he has cultivated the mosquito cycle of the malarial parasite, though his results have not yet been confirmed. There is no reason to doubt that the phases of development of protozoa in insects may be produced in the culture tube.

An abridged account of these researches, illustrated by Plate XXXIV, was published in the Proceedings of the Royal Society, B, Vol. 87 (1913), pp. 77-87. We have much pleasure in returning thanks to the Royal Society for permission to reprint Plate XXXIV.

We wish to thank Dr. H. B. Fantham for his very valuable help during our work on this subject.

CULTIVATION TECHNIQUE

Our method of cultivation is almost the same as that of Bass and Johns, except that it is less complicated. Ten c.c. of blood is drawn from a vein and transferred to a sterile test tube containing a thick wire leading to the bottom of the tube from the cotton wool plug. 1/10 c.c. of a 50 per cent. aqueous solution of glucose is added to this tube, preferably before adding the blood.

The blood is defibrinated by gently stirring with the thick wire. Defibrination should be complete in about 5 minutes. The wire with the clot is then removed and the blood is poured into several smaller sterile tubes (about one inch column of blood in each). A rubber cap is placed over the cotton plugs to prevent evaporation and the tubes are then transferred (standing upright) to an incubator at a temperature of 37° to 41° C. The corpuscles settle in a short time, leaving about one half-inch of clear serum at the top. It is apparently unnecessary to remove the leucocytes by centrifugalisation.

FURTHER OBSERVATIONS ON CULTIVATION

We have grown four complete generations of parasites in one tube by the above method, and we do not see why their growth should not continue indefinitely, provided fresh serum and corpuscles be added. It is not the presence of leucocytes which prevents further development. This is due to degenerative changes taking place in the corpuscles and serum. If the serum and corpuscles be kept in a sterile condition in an ice chest they remain unchanged for a long time; but at the temperature required for the growth of the parasites visible changes take place in a few days. The corpuscles become fragmented and form a brownish débris, and the serum becomes dark brown in colour. When this occurs the parasites are unable to continue their developmental cycle. Bass and Johns in their original paper (1912) stated that the parasites grow only on the surface layer of corpuscles, and that no growth took place in the deeper layers. They also stated that the serum destroyed the parasites when they escaped from the corpuscles, so that when the spores escape they must enter immediately into a contiguous corpuscle in order to survive. The blood also, in their opinion, required to be heated to a temperature of 40° C. to destroy the complement in the serum; furthermore, in order to cultivate several generations, the leucocytes had to be removed. We do not believe that they have sufficient evidence for these deductions. We find that the parasites develop even in the very deepest layer of the column of corpuscles. Also, it seems rather a contradiction that the parasites should grow best on the surface layer of corpuscles

next to the serum and the leucocytes which are supposed to destroy them. No doubt the leucocytes ingest some of the parasites, but they are never able to ingest all of them. Again, in one of our most successful cultures the temperature of the blood never exceeded 38° C., and we have found that they are able to grow at a temperature as low as 36° C. This is rather against the theory of complement destruction. In an able paper by Mary Rowley Lawson (1913), considerable evidence is brought forward to show that the parasites are extra-corpuscular during their entire development. If this is true, then the parasites would be constantly in contact with the serum which is supposed to destroy them. It appears to us that the only conditions necessary for the successful cultivation of the parasites are fresh corpuscles, fresh serum, a temperature of 37° to 41° C. and the requisite amount of glucose. The presence of leucocytes and complement is apparently immaterial.

THE OPTIMUM TEMPERATURE

In our opinion the optimum temperature for cultivation is about 38° C. On two occasions we incubated identical culture tubes from the same patient, some at 37° C. and some at 41° C. On both the occasions the parasites developed much more successfully in the tubes incubated at 37° C.

HAEMOLYSIS IN THE CULTURE TUBES

This is a rare occurrence, having been seen only once out of fifteen cases. A kind of haemolysis occurs after the blood has remained in the incubator for several days. The corpuscles degenerate into a brownish débris, and the serum shows a brown discoloration.

CLUMPING OF THE MALIGNANT TERTIAN PARASITES

This phenomenon has been observed in all our malignant tertian cultures (twelve cases). It occurs even when the parasites are scarce. It is best observed in wet films, as smearing of the blood tends to break up the clumps. No tendency to clumping occurred in our benign tertian cultures.

RESISTANT FORMS OF PARASITES

Sometimes it will be found that the parasites do not grow well *in vitro*. They may grow only partially, stopping short of segmentation. This is liable to occur if quinine has been given to the patient before the blood is drawn. In other cases, only a few of the parasites reach maturity, these apparently being able to resist successfully adverse conditions in the culture tube.

THE MORPHOLOGY OF *PLASMODIUM FALCIPARUM* IN CULTURE

The blood examined before incubation always showed the typical small ring parasites of malignant tertian malaria; these varied in size, with a maximum diameter of about 3μ. There was no enlargement of the red blood corpuscles, and no Schüffner's dots. Plate XXXIV, fig. 1, shows a small ring parasite from the peripheral blood before incubation. After many experiments in Liverpool it has been found that there is great variation in the rate of growth of *P. falciparum* in artificial media, and many suggestions can be offered to explain this phenomenon. J. G. Thomson and S. W. McLellan (1912) found in one case that maximum sporulation of *P. falciparum* occurred in twenty-five hours. This culture was made from a case with very heavy infection of parasites, and the patient had taken no quinine. The temperature of the incubator was only 38° C., but the glucose added was slightly in excess of that recommended by Bass (1912). The rapid segmentation of the plasmodia in this case, therefore, may have been due to two causes: (1) the age of the parasites when introduced into the culture tube, since it is to be noted that they were fairly large rings, about 3μ in diameter, and (2) the quantity of glucose may in some way have hastened the growth. In other experiments, however, where the glucose was slightly in excess there was no such rapid growth, so that in all probability the real cause of the rapid segmentation of parasites *in vitro* is due to the age of the plasmodia when drawn for incubation purposes. Another factor which seems to influence the rate of growth is the previous administration of quinine, and, in fact, this may inhibit the growth entirely. On several occasions we have made unsuccessful attempts

to cultivate the malignant tertain parasite, and these have usually been from patients to whom quinine had been administered.

On two occasions complete sporulation did not take place till after fifty hours' incubation at 41° C. On one of these occasions the parasites completed several generations, as illustrated by the accompanying coloured plate (Plate XXXIV, figs. 1-20). We found that maximum segmentation took place in fifty-two hours. Division of the chromatin into daughter nuclei began in about thirty-six hours (figs. 4 and 5). In forty-seven hours the number of spores had increased (figs. 6 and 7), and more or less complete segmentation took place in fifty-two hours. Fig. 11 shows a parasite which has produced thirty-two daughter cells, and these have broken loose from the corpuscle. It is to be noticed that in the peripheral blood the small ring parasites show no pigment. After incubation the rings gradually grow in size (fig. 3), and at a certain stage, immediately previous to the splitting of the chromatin a round compact mass of pigment appears, usually situated near one margin of the parasite, which now measures about 5μ or 6μ in longest diameter. In the culture under discussion this round mass of pigment was seen in thirty-six hours (figs. 4 and 5). J. G. Thomson and S. W. McLellan (1912) found that the pigment had collected in twelve hours. The parasites immediately previous to segmentation measure about 5μ to 6μ in their longest diameter, and all show the circular mass of compact pigment. We may call this stage the pre-segmenting stage. The chromatin now begins to split into two, and if segmentation is complete thirty-two daughter cells may be formed (fig. 11). All stages of segmentation can be found, from two spores up to thirty-two spores as a maximum. During segmentation the circular mass of pigment takes a central position, and the spores form in a circular arrangement around these (figs. 7 to 11). In this culture segmenting forms were found at different stages up to fifty-six hours, this being, no doubt, due to the fact that the parasites were not all of the same age when introduced into the culture tubes.

In seventy-five hours all segmenting forms had disappeared and only very young parasites were found, about 1·5μ in diameter and containing no vacuole (fig. 13). This represents the beginning of a second generation. It is to be concluded, therefore, that many

of the young merozoites escape ingestion by the leucocytes and enter a new red cell. The same culture examined in three days again showed segmenting forms (fig. 14); but in this case the spores were never so numerous as 32, and this can easily be explained by the adverse conditions which have now developed in the culture tube. These conditions did not, however, prevent the young parasites attempting to segment. In four and a half days (fig. 15) we again only found young rings, which represent the beginning of a third generation; and in six days sporulating forms were again found (fig. 16). Here, only eight spores have formed, and it was evident that the power of the parasite to undergo full segmentation was getting gradually less. On the seventh day, again, only very young plasmodia were found, which represented a fourth generation, and these again attempted to sporulate (figs. 18 and 19) on the eighth and ninth days respectively. On the tenth day only young parasites again were found, which represented a fifth generation. No further development, however, occurred, as the conditions in the culture were gradually becoming more and more adverse. Thus we have evidence that the parasites will, under very suitable conditions, actually proceed in the original culture tube through four complete generations without the medium being in any way renewed.

We now wish to draw particular attention to several points of great interest which occur in the cultures of *P. falciparum*. In these there is a definite tendency for the parasites to clump together into masses immediately the circular mass of pigment appears, and even before segmentation begins. A mass of seven parasites is seen in the microphotograph, Plate XXXV, fig. 2. This photograph was taken after twelve hours' incubation at 38° C. In all of these note the dark circular mass of pigment, the parasite having a diameter of about 6μ. At this stage the chromatin is dispersed into the protoplasm of the plasmodium, and as yet shows no division into daughter cells. This tendency to clump becomes more marked when sporulation actually begins, and fig. 3, Plate XXXV, shows a large mass of sporulating parasites, the photograph being taken after twenty-five hours' cultivation. This picture shows all stages of sporulation and illustrates in a very striking manner the tendency of the parasites to agglomerate into masses. This clumping in

malignant tertian malaria was seen by Dr. Cropper (1908) in the peripheral blood of a patient in Jerusalem, and with his kind permission we have been able to reproduce a microphotograph (Plate XXXV, fig. 5) of his plate in the 'Lancet,' which shows a mass of these pre-segmenting parasites, each with a circular mass of pigment. This must have been a most unusual case, as these forms are seldom seen in the peripheral blood in malignant tertian malaria; but it is of extreme interest, since it proves the tendency of the parasites to clump together during the pre-segmenting stage of development. When we compare this figure of Dr. Cropper's, photographed from the peripheral blood of a case of malignant tertian malaria (fig. 5), with our own microphotograph, obtained from a culture twelve hours old (fig. 2), we are at once impressed with this phenomenon; and it is to be noted that this tendency to clump remains until full sporulation. Fig. 3 shows a whole field of a microscopic preparation filled with segmenting forms. In our opinion this phenomenon explains why the sporulation of *P. falciparum* occurs, as a rule, only in the capillaries of the internal organs. All the large parasites tend to get caught in the fine capillaries of the internal organs, and thus only young rings, whose containing corpuscles have not yet acquired the clumping tendency, are seen in the peripheral circulation. This fact explains many phenomena of pernicious malaria, and shows how comatose malaria may occur in infections with *Plasmodium falciparum*, in which condition the brain capillaries are filled with sporulating parasites. Why Dr. Cropper's case showed pre-segmenting forms and also segmenting forms in the peripheral blood is uncertain, but it could well be suggested that these were the overflow from fine capillaries of the internal organs which were already packed with parasites, and could not hold more. It has been observed by us that segmenting forms of *P. falciparum* are only found in the peripheral blood of patients suffering from extremely heavy infections.

THE MORPHOLOGY OF *PLASMODIUM FALCIPARUM* IN THE HUMAN HOST

This is identical in every respect with that obtained *in vitro*, so that it is hardly necessary to describe it in detail. As already stated, only the young ring forms of this parasite are found, as a

rule, in the peripheral blood. This is due to the fact that when these parasites have grown larger than the ring stage, the containing corpuscles stick together, forming clumps which are unable to circulate, and thus are arrested in the fine capillaries of the inner organs. The study of the further stages of this parasite is obtained by examining smears of the inner organs, such as the spleen, liver, brain, etc., of deceased patients. The autopsy smears of over one hundred cases have recently been examined by one of us (D. T.) in conjunction with Dr. W. M. James, in Panama (the results of this work will be published later). In such smears the parasite is found in different stages of development. In some, pre-segmenters only are found; some show only young rings, and others only sporulating forms. This depends entirely on the stage of development of the parasite at the time of the patients' death. In some cases, of course, several stages of development are found, since one patient may contain several broods of parasites which are in different stages of development. In only one case did we find the parasites in the stage of maximum sporulation. All of them contained over 20 spores, and 13 per cent. had 32 spores. One of these is represented in the microphotograph (Plate XXXV, fig. 4). We think that there can be little doubt that we have proved conclusively, both from observations in the culture tube and in the human host, that *P. falciparum* is capable of producing a maximum of 32 spores under favourable circumstances. This is a much larger number than has been previously given in the writings of competent observers. Ross (1912) quotes the figures of Welch (1897) at 6 to 20 or more spores. Later, however, from observations made by himself and one of us, he taught that the maximum number was 2^5 or 32. Marchiafava, Bignami and Mannaberg (1894) state that the numbers vary from 8 to 15, while the following authors give the numbers as follows. Stephens and Christophers (1908) 8 to 10, Deaderick (1910) 5 to 25 and even 30, Gulland and Goodall (1912) 8 to 15. It is probable that these discrepancies are due to two reasons: (*a*) observations of autopsy smears in which the sporulation had not reached its full maturity, and (*b*) observations on autopsy smears of patients to whom quinine had been given before death. In such cases incomplete and atypical sporulation is seen. We do not claim,

of course, that 32 spores are always produced, even under favourable conditions, without quinine administration. It is likely that the numbers produced vary considerably, just as in the case of benign tertian, which produces numbers varying from 16 to 26. The clumping phenomenon in the human host is represented in Plate XXXV, fig. 5. This clump was, as already stated, found by Dr. Cropper in a peripheral blood smear. A similar phenomenon is seen in the brain capillaries of cases of comatose malaria.

THE MORPHOLOGY OF *PLASMODIUM VIVAX* IN CULTURE

Here we are studying a parasite in which all stages, from small rings up to full sporulation, may be seen in the peripheral blood. Sporulation does not necessarily occur in the internal organs, and it is quite usual to find these forms in ordinary peripheral blood smears. When cultivating this parasite, therefore, it is important to draw the blood from the patient when the young forms predominate, so that it is then certain whether or not we are obtaining further development in the culture tubes. In the culture illustrated in Plate XXXIV, figs. 21 to 30, we obtained the blood when young rings (fig. 21) predominated, and no segmenting forms were found. After eight hours' incubation at a temperature of 39° C., a marked increase in size of the parasites was noted, figs. 22, 23 and 24, and pigment was now evident, being scattered throughout the protoplasm in fine granules. This arrangement of the pigment in *P. vivax* is in marked contrast to what occurs in *P. falciparum*, where the pigment always becomes arranged in a dense circular mass from the commencement. After 20 to 29 hours' incubation (figs. 25-30), sporulation was seen at different stages, the pigment being collected into a loose mass of granules in the centre of the parasite. Fig. 29 shows a parasite with 15 daughter cells, and we have found on several occasions sixteen spores, which we think is the usual maximum of daughter cells found in *P. vivax*, although again we find all stages, from two spores up to sixteen, according to the stage of development at which we examine the cultures. We are quite certain that the spores are never so numerous as in the malignant tertian parasite. In these cultures clumping has not been found, and this explains why the parasites do not tend to be

arrested in the internal organs during sporulation, although when full grown they are much larger than the malignant tertian parasite.

The absence of clumping in the case of the benign tertian parasite explains satisfactorily the lack of pernicious symptoms in this infection, and, hence, the absence of comatose malaria, and it also explains why all stages of this parasite are found in the peripheral blood, even up to sporulation.

THE MORPHOLOGY OF THE BENIGN TERTIAN PARASITE IN THE HUMAN HOST

This is identical with that which we have just described under morphology in the culture tubes (Plate XXXIV, figs. 21-30). The chief features in which this parasite differs from *P. falciparum*, are the large size of the containing corpuscle with the presence of Schüffner's dots, the straggling form of the medium-sized parasites, the scattered pigment in the pre-segmenting stages and the smaller number of spores. These spores are larger than the spores of the malignant tertian parasite. The pigment in the sporulating forms is collected into a loose mass. With regard to the number of spores produced by this parasite, we have found as many as twenty-four, but the most usual number is 16 to 18. In culture the largest number we obtained on three occasions was 18. The following are the numbers of spores produced by *P. vivax* in the human host, according to several observers. Ross (1910) quotes the figures of Grassi and Feletti, 15 to 20 spores. Marchiafava, Bignami and Mannaberg (1894), 16 spores. Golgi quotes 14 to 19 spores. Stephens and Christophers (1908), 15 or more spores. Deaderick (1910) gives 12 to 26 spores, most often 16 spores. In our opinion Deaderick's figures are most correct. All stages of this parasite are found in the peripheral blood, and the phenomenon of clumping has never been observed. Ross has taught recently that there are four splits with this parasite—that is, sixteen spores; three splits with the quartan parasites—that is, eight spores; and five splits, or thirty-two spores, with the malignant parasite.

SUMMARY

(1) The malignant tertian parasite has been successfully cultivated, after the method of Bass and Johns, on twelve occasions, and the benign tertian parasite on three occasions by the present writers.

(2) It is unnecessary to remove the leucocytes from the blood before incubation. The optimum temperature would appear to be about 38° C., and the parasites may grow successfully at a temperature of 36° C. or 37° C.

(3) The time required for the full development of the parasite *in vitro* varies, but this variation is partly due to the age of the parasite at the time of incubation.

(4) The cultures of benign tertian differed from those of malignant tertian in that there was no tendency to clumping of the parasites in the former, either before or during sporulation.

(5) This difference appears to us to explain in a satisfactory manner why only young forms of malignant tertian are found in the peripheral blood, as the clumping tendency of the larger forms causes them to be arrested in the finer capillaries of the internal organs. It also explains the tendency to pernicious symptoms, such as coma, in malignant tertian malaria. All stages of the benign tertian parasite are found in the peripheral blood, and there are seldom pernicious symptoms, because there is no tendency to clumping.

(6) The malignant tertian parasite (*P. falciparum*) is capable of producing, in maximum segmentation, thirty-two spores. On the other hand, benign tertian (*P. vivax*) produces, as a rule, during maximum segmentation, sixteen spores; sometimes more may be produced, but the number is never thirty-two.

(7) The pigment in *P. falciparum* collects into a definite, circular, and very compact mass early in the growth of the parasite. On the other hand, during the growth of *P. vivax* the pigment remains scattered in definite granules throughout the body of the parasite, till just before segmentation, when it collects into a loose mass of granules in the centre of the full-grown *Plasmodium*.

(8) The morphology of *P. falciparum* and *P. vivax* in the human host is identical with the morphology of these parasites, as obtained in the culture tube.

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EXPLANATION OF PLATES

PLATE XXXIV

All the figures in this plate represent the growth of the parasites in the culture tube. The figures were drawn with an Abbé camera lucida from stained preparations. Magnification 1,600 diameters.

Fig. 1 is a malignant tertian parasite at the time of inoculation of the culture tube. Corpuscle is shrunken. No pigment is seen and no stipulation of the corpuscle.

Fig. 2 represents 12 hours' growth at 36° C. No pigment is yet visible.

Fig. 3 represents 23 hours' growth at 41° C. Pigment is not yet evident, but the parasite has increased in size.

Figs. 4 and 5 represent 36 hours' growth; note the appearance of a compact mass of pigment. Fig. 5 is a double parasite.

Figs. 6 and 7 show commencing segmentation after 47 hours' incubation. Fig. 6 shows five daughter cells, and fig. 7 twenty spores.

Figs. 8, 9 and 10 show sporulating forms after 51 hours' incubation.

Fig. 11 shows complete sporulation (32 spores) after 52 hours' incubation. The containing corpuscle has burst and liberated the spores.

Fig. 12 was obtained after 56 hours' incubation.

Fig. 13 represents a young merozoite of the second generation which has entered a new corpuscle after 75 hours' incubation. Note that there is no vacuole. The parasite is only 1·5μ in its longest diameter.

Fig. 14 represents sporulation of the second generation after 3 days' incubation.

Fig. 15 shows a young merozoite of the third generation after $4\frac{1}{2}$ days' incubation.

Fig. 16 represents sporulation in the third generation after 6 days' incubation. Only eight spores have formed.

Fig. 17 is a young merozoite of the fourth generation after 7 days' incubation.

Fig. 18. Eight days' incubation, showing commencing segmentation in the fourth generation.

Fig. 19. Nine days' incubation, showing sporulation in the fourth generation.

Fig. 20 shows a young merozoite of the fifth generation after 10 days' incubation.

All of the above figures were obtained from one culture tube, without the removal of leucocytes and without the addition of fresh serum or corpuscles.

Fig. 21 shows a young benign tertian parasite at the time of inoculation of the culture tube. The culture was made at the period when these young rings predominated. Note the large size of the corpuscle and the Schüffner's dots.

Figs. 22, 23 and 24 represent 8 hours' growth in culture tube at 39° C. The parasites have now distinctly increased in size, and fine granules of pigment are now seen scattered in the protoplasm.

Figs. 25-30 show parasites obtained from the culture tube after 20 to 29 hours' incubation. Fig. 25 shows four masses of chromatin.

Fig. 30 is a female gamete (undivided chromatin and scattered pigment) found in the culture tube; similar gametes were found in the blood at the time of inoculation of cultures, so it is probable that this gamete was one of these and that it had not developed in the culture.

Note the large size of the corpuscles in the benign tertian as compared with those in the malignant tertian; also the Schüffner's dots and the scattered pigment. The spores also are larger and fewer in number in the case of the malignant tertian parasites.

Plate XXXV

Microphotographs of malignant tertian parasite. Figs. 1 to 3 are from the same culture of *Plasmodium falciparum*, and are all photographed at the same magnification, namely, 1,500 diameters.

Fig. 1. A photograph of the malignant tertian parasite as seen in the peripheral blood before incubation. No pigment is seen. The small dark mass is the chromatin.

Fig. 2. The malignant tertian parasite after 12 hours' incubation at a temperature of 37° C. to 38° C. The parasites have grown to at least twice the size. A circular mass of compact pigment is now evident. This photograph illustrates the clumping tendency and seven parasites are seen grouped together. No division into daughter cells has yet taken place. The dark circular area is pigment, and the darker area, towards the margins, is due to the chromatin.

Fig. 3. The malignant tertian parasite after 25 hours' incubation at a temparature of 37° C. to 38° C. This photograph shows well the tendency to clump and the parasites are now seen at all stages of sporulation.

Fig. 4. Shows two parasites from a smear of the spleen showing segmentation. Thirty-two spores can be counted in the one in the centre of the field.

Fig. 5. This is a photograph taken from a coloured plate drawn by Dr. Cropper and shows the clumping of the red cells containing pre-segmenting forms of the malignant tertian parasites. This drawing was made from a smear of the peripheral blood (*vide* 'Lancet,' July 4th, 1908, and coloured plate fig. 1). (Magnification about 1,000 diameters).